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REPORT

Experimental validation of a predicted feedback loop in the multi-oscillator clock of *Arabidopsis thaliana*

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Our computational model of the circadian clock comprised the feedback loop between *LATE ELONGATED HYPOCOTYL (LHY)*, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* and *TIMING OF CAB EXPRESSION 1 (TOC1)*, and a predicted, interlocking feedback loop involving *TOC1* and a hypothetical component *Y*. Experiments based on model predictions suggested *GIGANTEA (GI)* as a candidate for *Y*. We now extend the model to include a recently demonstrated feedback loop between the *TOC1* homologues *PSEUDO-RESPONSE REGULATOR 7 (PRR7)*, *PRR9* and *LHY* and *CCA1*. This three-loop network explains the rhythmic phenotype of *toc1* mutant alleles. Model predictions fit closely to new data on the *gi;lhy;cca1* mutant, which confirm that *GI* is a major contributor to *Y* function. Analysis of the three-loop network suggests that the plant clock consists of morning and evening oscillators, coupled intracellularly, which may be analogous to coupled, morning and evening clock cells in *Drosophila* and the mouse.

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Introduction

The circadian clock generates 24-h rhythms in most eukaryotes and in cyanobacteria (Dunlap *et al*, 2003), including the rhythmic expression of 5–15% of genes in eukaryotes (Duffield, 2003). Circadian rhythms are generated by a central network of 6–12 genes that form interlocked feedback loops (Glossop *et al*, 1999). The relatively small number of components involved in the circadian clock network makes it an ideal candidate for mathematical modelling of complex biological regulation (Ruoff and Rensing, 1996; Leloup and Goldbeter, 1998; Forger and Peskin, 2003).

The clock mechanism in the model plant, *Arabidopsis thaliana*, was first proposed to comprise a feedback loop in which two partially redundant genes, *LATE ELONGATED HYPOCOTYL (LHY)* and *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, repress the expression of their activator, *TIMING OF CAB EXPRESSION 1 (TOC1)* (Alabadi *et al*, 2001). This circuit cannot fit all experimental data (Locke *et al*, 2005a), as a short-period rhythm persists for several cycles both in *lhy;cca1* (Alabadi *et al*, 2002; Locke *et al*, 2005b) and in

toc1 mutant plants (Mas *et al*, 2003a). Previously we used mathematical modelling to propose a new circuit comprising two interlocking feedback loops in order to explain the residual rhythm in the *lhy;cca1* plant (Locke *et al*, 2005b). This model predicted the existence and expression patterns of two hypothetical components *X* and *Y*. *X* is proposed to be activated by *TOC1*, and *X* protein then activates *LHY* transcription, as required by the expression profile of *TOC1* protein (Mas *et al*, 2003b). *Y* forms a second loop with *TOC1*, which is responsible for the short-period oscillation in the *lhy;cca1* mutant. Based on the similarity of predicted and observed expression patterns, *GI* was identified as a candidate for *Y* (Locke *et al*, 2005b).

Here we have extended our model to include the recently proposed feedback loop between *PSEUDO-RESPONSE REGULATOR 7 (PRR7)*, *PRR9* and *LHY/CCA1* (Farre *et al*, 2005; Salome and McClung, 2005), resulting in a three-loop circuit (Figure 1A). We first validate this new model against existing and new experimental data. We then experimentally confirm our prediction that *GI* functions as a component of *Y* in a feedback loop with *TOC1*, and investigate the regulatory properties of the three-loop network.

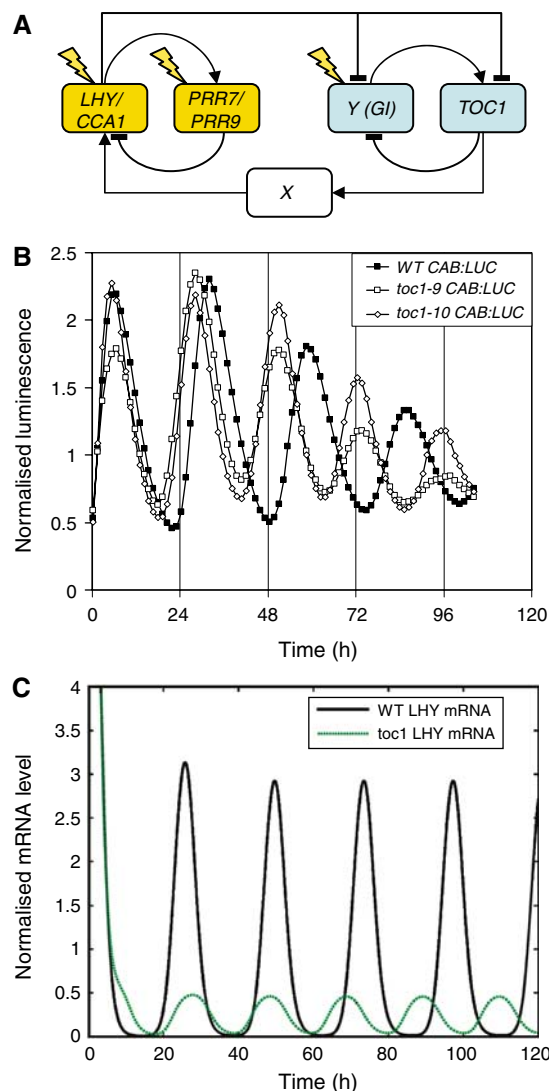


Figure 1 The three-loop *Arabidopsis* clock model accounts for 20 h rhythms in *toc1* mutants. **(A)** Summary of the three-loop network, showing only genes (boxed), regulatory interactions (arrows) and the locations of light input (flashes). Two-component oscillators are distinguished by shading the gene names in yellow or blue. **(B)** *CAB:LUCIFERASE* (*CAB:LUC*) rhythms in WT (filled squares), *toc1-9* (open squares) and *toc1-10* (open diamonds) under constant red light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$). Luminescence values were normalised to the average over the whole time course. Time zero is the onset of constant light (LL). **(C)** Simulated expression levels of *LHY* mRNA in the WT (black solid line) and *toc1* backgrounds (green dotted line) in LL. Expression levels were normalised to the average level of expression. Translation rate of *TOC1* mRNA in the simulated mutant is 1/1000 WT value.

Results

A three-loop clock network accounts for additional experimental data

A short-period rhythm can exist in mutants with reduced *TOC1* function in some conditions (Alabadi *et al*, 2001; Mas *et al*, 2003a). In order to test whether such residual rhythmicity was due to residual wild-type (WT) *TOC1* mRNA, we tested a *TOC1* deletion mutant (Supplementary information) for rhythmic expression of *CHLOROPHYLL A/B-BINDING PROTEIN2* (*CAB2*,

also known as *LHCB1*1*), a morning-expressed clock output gene (Figure 1B). Plants of the Ws accession carrying the *toc1-10* deletion had a rhythm of 20 h period and reduced amplitude under constant light (LL) conditions. This was identical in timing to the rhythm of *toc1-9* plants, which carry a termination codon within the first domain of the predicted *TOC1* protein. Taken together, these data confirm previous suggestions that a *TOC1*-independent oscillator can persist in *toc1* plants (Mas *et al*, 2003a).

The proposed *PRR7/PRR9-LHY/CCA1* feedback loop provided a candidate mechanism to account for this oscillation. We therefore added this loop to the interlocked feedback model (Figure 1A; Supplementary information) to create a three-loop model. As the mutant phenotypes of *PRR7* and *9* are weak, apparently less than 1 h different from WT (Nakamichi *et al*, 2005), we grouped these genes together as one gene, *PRR7/9*, in our network equations (Supplementary information). *LHY* and *CCA1* were grouped together as *LHY* (Locke *et al*, 2005b). The first feedback loop involves *LHY* activating *PRR7/9* transcription (Farre *et al*, 2005), with *PRR7/9* protein going on to repress *LHY* activation. The remainder of the network follows our previous model (Locke *et al*, 2005b). *LHY* represses *TOC1* and *Y* transcription; the dual, repressing and activating role of *LHY* has experimental support (Harmer and Kay, 2005). *TOC1* protein activates *X* transcription, with *X* activating *LHY* transcription to form a second feedback loop. *Y* activates *TOC1* expression and *TOC1* represses *Y* expression, forming the third feedback loop. Light activates expression of *LHY*, *Y*, and now also *PRR7/9*, because *PRR9* has been shown to be acutely light-activated (Ito *et al*, 2003).

We used an extensive parameter search for the new and altered components to test whether the three-loop network could account for the residual oscillations of a *toc1* deletion mutant (Supplementary information). Our simulations show that the *PRR7/PRR9-LHY/CCA1* loop can generate the short-period rhythm of *toc1* plants (Figure 1C), and its absence can result in the very long period of *prp7:prp9* double mutants (Supplementary Figure 1; Farre *et al*, 2005). Neither of these observations could be accounted for with our interlocked feedback loop model (Locke *et al*, 2005b), which predicted arrhythmia or a long period under all conditions in simulations of a *toc1* null or loss-of-function mutants such as *toc1-2* (6% of WT RNA levels; Strayer *et al*, 2000) or the *toc1* RNAi lines (10–15%, Mas *et al*, 2003a). Sensitivity analysis shows that the three-loop model is similarly tolerant of parameter changes as the interlocking-loop model (Supplementary information; Supplementary Figure 2).

We now use the more realistic three-loop model to make further predictions for *Y*'s role in the clock, and test these predictions against the experimental manipulation of *GI*.

GI is a component of *Y*

A simulated *gi* mutation (modelled by reducing *Y* translation by 70%) gives a 1 h reduction in the period of *LHY* mRNA oscillations (Figure 2A), which matches well with the observed period of *CAB* expression rhythms in a *gi* null mutant background (Figure 2B). According to our models, the *Y-TOC1* feedback loop generates the 18 h rhythm seen in an *lhy:cca1* mutant (Figure 2C and D). A reduction in *Y* function in

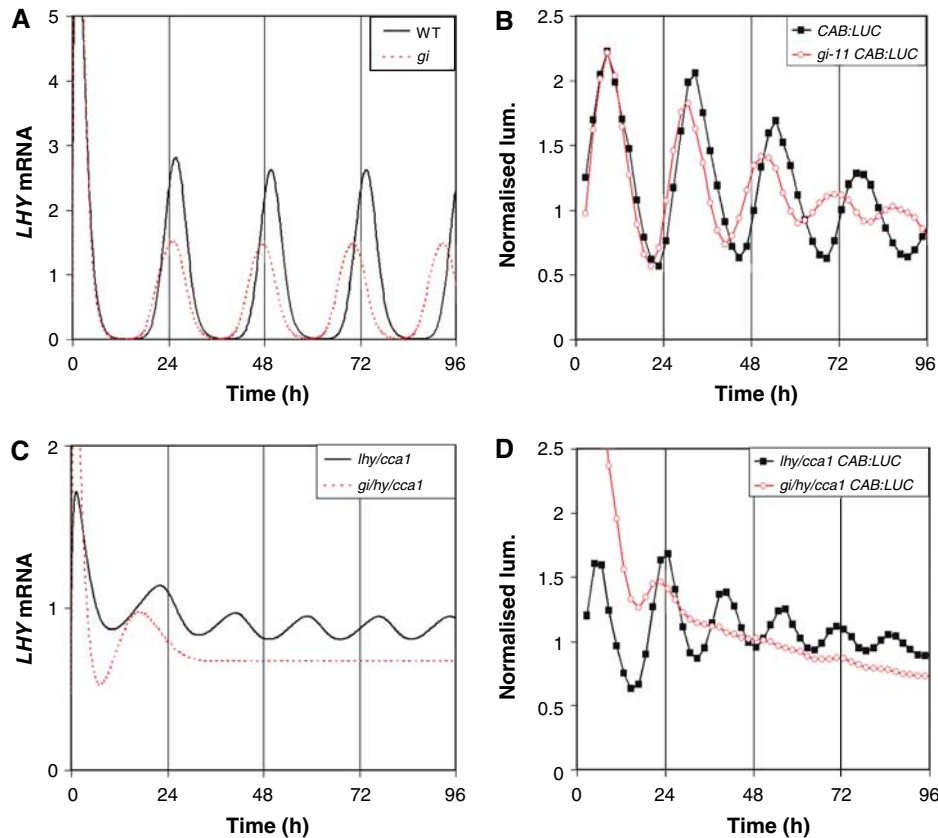


Figure 2 *GI* acts as *Y* in a feedback loop with *TOC1*. (A) Simulation of *LHY* mRNA levels in the WT (black solid line) and *gi* backgrounds (*Y* translation rate reduced by 70%, red dotted line) under constant light (LL). (B) Corresponding experimental data assaying circadian control of WT *CAB:LUC* expression by video imaging. (C) Simulation of *LHY* mRNA under LL in *lhy;cca1* (translation rate of *LHY* mRNA in simulated mutant is 1/1000 WT value, black line) and *gi;lhy;cca1* mutants (red dotted line). (D) Corresponding experimental data assaying *CAB:LUC* expression. The *gi;lhy;cca1* mutant is severely damped (only four out of 23 plants gave a period estimate within the circadian range, and those estimates had an average relative amplitude error of 0.86). All data were normalised to the average level of expression.

the *lhy;cca1* mutant background should therefore reduce the robustness of this residual rhythm. In fact, simulation of the *gi;lhy;cca1* triple mutation results in a rapid loss of rhythmicity, reaching a negligible amplitude during the second cycle in LL (Figure 2C). The very strong phenotype encouraged us to test the rhythms of *gi;lhy;cca1* triple mutant plants (Figure 2D). An almost exact match is made to the simulation; in the *gi;lhy;cca1* triple mutant, the rhythmic amplitude collapses to insignificance during the second cycle.

An identical, catastrophic damping is also seen experimentally in the rhythmic expression of *TOC1* and of *COLD AND CIRCADIAN REGULATED 2* (*CCR2*), an evening-expressed clock output gene, in the triple mutant under LL and constant darkness (DD) (Supplementary Figure 3), whereas the *lhy;cca1* double mutant retains short-period rhythms as described (Locke *et al*, 2005b). The mean level of *TOC1* expression is significantly reduced in the *gi;lhy;cca1* triple mutant compared with the *lhy;cca1* double mutant (Supplementary Figure 4A). This is consistent with *GI*'s functioning in the predicted role of *Y*, activating *TOC1*, and matches well to the expression levels in the simulated double and triple mutants (Supplementary Figure 4B). Our predictions also fit with experimental work showing that *GI* expression is light-responsive (Fowler *et al*, 1999; Paltiel *et al*, 2006), and are consistent with *GI* function in

balancing other clock components to generate temperature compensation (Gould *et al*, 2006). *GI* is a component of a light-activated feedback loop, separate from *LHY* and *CCA1*, which is required for the maintenance of residual rhythms in the *lhy;cca1* background.

Morning and evening oscillators allow tracking of dawn and dusk

Our three-loop model suggests a symmetrical structure for the *Arabidopsis* clock circuit. The model predicts that two short-period oscillators, the morning-expressed *PRR7/9-LHY/CCA1* loop and the evening-expressed *TOC1-Y/GI* loop, are coupled together by the *LHY/CCA1-TOC1-X* loop (Figure 1A). We investigated the effect of a change of photoperiod on the phase of the clock components of our three-loop network (Figure 3). The clock-regulated expression of *LHY* mRNA before dawn (20–24 h) remains at a fixed phase relative to dawn. In contrast, the peak of *TOC1* mRNA is delayed under long photoperiod conditions, showing that its phase also responds to the time of dusk. This flexibility is not seen in our one-loop or interlocked-loop models (Supplementary Figures 5 and 6), in which clock-regulated *LHY* and *TOC1* expressions are fixed

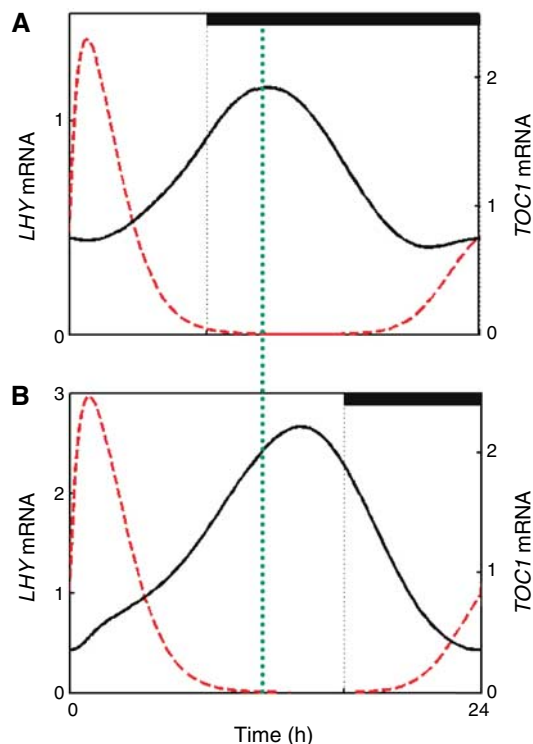


Figure 3 Three-loop network can track dawn and dusk. Simulations of *TOC1* mRNA (black solid line) and *LHY* mRNA (red dotted line) using the three-loop network under photoperiods of (A) LD8:16 and (B) LD16:8. The vertical dotted line highlights the shift in the peak phase of *TOC1* mRNA levels from LD8:16 to LD16:8. The peak phase of *LHY* mRNA is not shifted.

relative to dawn, or both move with the time of dusk. Note that *LHY* is light-induced in all the models, so its peak phase is forced by dawn. The three-loop structure of the clock provides the flexibility to track multiple phases (Rand *et al*, 2004).

The three-loop model also predicts that, if the coupling between *PRR7/9*–*LHY/CCA1* loop and the evening-expressed *TOC1*–*Y/GI* loop were impaired, the two oscillators might run with different periods within one cell. This is predicted by simulation of an *x* mutant (Supplementary Figure 7), where *LHY* mRNA levels oscillated with a 20.4 h period under LL conditions and *TOC1* levels oscillated with a 17.3 h period.

Discussion

We present evidence that *GI* acts with *TOC1* in a feedback loop of the circadian clock in *A. thaliana*. This marks an advance in systems biology, because *GI* was identified as a candidate gene in this loop using experiments based directly on predictions from mathematical modelling. The three-loop model has greater realism, as it can simulate the short-period rhythms of *toc1* and *gi* mutant plants and the long-period rhythms of *prp7;prp9* double mutants, while still correctly matching the mutant phenotypes accounted for by the previous model. Understanding the *Arabidopsis* clock as a system of coupled, morning and evening oscillators provides a new intellectual framework that may persist over multiple incremental advances in biochemical and genetic realism.

The three-loop model is not yet complete, as it does not incorporate known clock-affecting genes such as *PRR3*, *PRR5*, *TIME FOR COFFEE (TIC)*, *EARLY FLOWERING 4 (ELF4)* and *LUX ARRHYTHMO (LUX)* (reviewed by McClung, 2006). Rather than a weakness, this indicates three important uses of even incomplete mathematical models, in providing a framework to understand the existing experimental results, in focusing future experimental work on key regulatory interactions that reveal the location of the additional genes within the network and in informing the detailed design of these experiments, specifically to test any unusual aspect of regulation that has been predicted by simulation (Locke *et al*, 2005b).

The three-loop circuit contributes to the apparent robustness of the *Arabidopsis* clock, along with the partial redundancy of some genes: few single mutations alter the clock period by more than 3–4 h and arrhythmic mutations are rare (McClung, 2006). *GI*, one of the first characterised clock-affecting genes (Fowler *et al*, 1999; Park *et al*, 1999) with complex functions in both flowering and circadian regulation (Mizoguchi *et al*, 2005; Gould *et al*, 2006), illustrates the difficulty of understanding the effect of one component upon a complex network. The *gi* single mutant had a relatively weak phenotype, whereas our assays of the triple *gi;lhy;cca1* mutant demonstrate *GI*'s importance (Figure 2 and Supplementary Figure 3) as one component of *Y* in the three-loop network. It is likely that other components participate in the evening feedback loop with *TOC1*, because our current model indicates that the circadian phenotypes of the *gi* single mutant and the *gi;lhy;cca1* triple mutant are accurately simulated by a 70% reduction in *Y* translation, rather than a complete absence of *Y* (Figure 2 and Supplementary Figure 3). *PRR5* is a candidate component of *Y* that should now be tested, perhaps in combination with the *gi* mutation. If *PRR5* is indeed part of *Y*, then our model could explain the arrhythmicity of the *prp7;prp9;prp5* triple mutant (Nakamichi *et al*, 2005): the triple mutation not only removes the *PRR7/9* feedback loop, but also impairs the *TOC1*–*Y* feedback loop. Constructing such multiple mutants, in combination with reporter genes, is and will remain laborious. Insertional mutants in most *Arabidopsis* genes are publicly available, but there is no prospect of a comprehensive bank of double mutants. Modelling offers a crucial tool for targeting future mutant construction as well as for extracting the maximum value from time-series studies using existing genetic resources.

Analysis of the three-loop network suggests new avenues for experiments. For example, the prediction that an *x* mutation could lead to desynchronisation of two short-period clocks (Supplementary Figure 7) suggests that future research could target mutations or chemical manipulations that cause desynchronisation of *LHY* and *TOC1* mRNA rhythms. Period differences among rhythms in the same plant have been observed repeatedly and in some cases can be interpreted as evidence for desynchronisation of two intracellular oscillators, although cell-type-specific effects cannot be excluded (Hall *et al*, 2002; Michael *et al*, 2003). The three-loop model provides a mechanism for such intracellular desynchronisation, if the various rhythmic processes are controlled by different loops and coupling between loops is weakened in some conditions. This flexibility of circadian regulation is expected to offer a

selective advantage, particularly where seasonal changes in photoperiod vary the relative timing of dawn and dusk (Pittendrigh and Daan, 1976). There is strong evidence in *Drosophila* (Stoleru *et al*, 2004) and mammals (Jagota *et al*, 2000) for separate control of morning and evening processes by oscillators in different cells, which are coupled together by cell–cell signalling. Plant clocks are coupled only weakly between cells, if at all (Thain *et al*, 2000), but the three-loop circuit suggests that an analogous architecture can be constructed within a single cell, by coupling the loop of morning-expressed genes *LHY/CCA1* and *APPR7/9* to the evening-expressed *TOC1-GI* loop. It will now be important to understand the role and balance of the light inputs into each of the feedback loops of the clock, firstly to determine what flexibility the three-loop circuit could provide and then to understand how the plant has evolved to exploit this flexibility in controlling rhythmic functions at different times of day.

Note added in proof

Zeilinger *et al*, in a study published simultaneously in *Molecular Systems Biology*, add PRR7 and PRR9 in parallel feedback loops to the interlocked loop network, with an alternative parameter set and light input mechanisms to PRR9 and Y (Zeilinger *et al*, 2006).

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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